Analysis for the Influence of the First Intron of Human CYP1A1 Gene on its AhR–mediated Transcription by using an IRES-Connected Luciferase Reporter System

Yoshinori Sakata[1], Seiichiroh Ohsako[2]

[1]Graduate School of Global Environmental Studies, Kyoto University
[2]National Institute for Environmental Studies

Introduction

Cytochrome P450 1A1 (CYP1A1) is the microsomal enzyme responsible for the bio-activation of carcinogenic compounds, and is known to be induced by aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene (3-MC). The *CYP1A1* gene is actively regulated by the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor. Exposure to TCDD results in numerous pathophysiological abnormalities, immune dysfunction and several types of cancer, and most of these effects are mediated by AhR. Moreover, *CYP1A1* knockout mouse experiments suggest that CYP1A1 contributes to lethality, uroporphyria, wasting syndrome and hepatocyte hypertrophy.[1] Therefore, detailed analyses of the CYP1A1 induction mechanism will be important to develop a clear understanding of TCDD-induced toxicity.

CYP1A1 gene transactivation is enhanced by the binding of AhR to xenobiotic responsive elements (XREs) clustering within the 1500-bp upstream region of the exon I of *CYP1A1*. However, the stable transformants with the reporter gene connected to a 1500-bp 5'-flanking region of *CYP1A1* did not mimic the exact pattern of endogenous CYP1A1 mRNA expression, i.e., maximum induction levels.[2] This suggests that DNA sequences that could be located further upstream, downstream, or within *CYP1A1* itself might regulate the maximum induction levels of CYP1A1 mRNA.

In the present study, we examined the influence of the first intron of *CYP1A1* composed of a relatively large (2500 bp) region on its ligand-AhR mediated transcription activity. To make a comparison between the transcriptional levels with and without the first intron of *CYP1A1*, we used the IRES (internal ribosome entry site) sequence to generate reporter vectors which coexpress partical CYP1A1 mRNA and firefly luciferase mRNA.

Materials and Methods

Plasmids The CMV immediate-early enhancer/promoter of pCI-neo vector (Promega, WI) was removed between restriction sites *Bgl* II and *Mlu* I, and the oligonucleotides (sense: 5'-GATCTACGCG TTTATTAGGC CGGCCGAATT CTAGGTGAGT AACTCGAGA-3', anti-sense: 5'-CGCGTCTCGA GTTACTCACA CCTAGAATTC GGCCGGCCTA ATAAACGCGTA-3') were inserted. The IRES sequence from pIRES-hrGFP-1a vector (Stratagene, CA) and the luciferase coding region from pGL3-Basic vector (Promega) were connected via the *Nco* I site and then inserted into the modified pCI-neo vector by *Xho* I and *Xba* I sites to generate pIRESluc. A human CYP1A1 DNA gene fragment was generated by PCR amplification using human genomic DNA (BD Biosciences Clontech, CA). PCR was performed using TaKaRa La Taq™ (TaKaRa Bio Inc, Shiga, Japan). The 2029 bp within the first intron (+304 ~ +2332, 87% of the first intron) were deleted by the *Xba* I sites. Five separate parts of *CYP1A1* DNAs were then inserted into pIRESluc to generate 5 reporter constructs as shown in Fig 1 (pIRESluc-hcype1; -1524 ~ +42, pIRESluc-hcype2; -1524 ~ +2751, pIRESluc-hcype2x; -1524 ~ +304 and +2333 ~ + 2751, pIRESluc-hcype7; -1524 ~ +4931, pIRESluc-hcype7x; -1524 ~ +304 and +2333 ~ +4931).

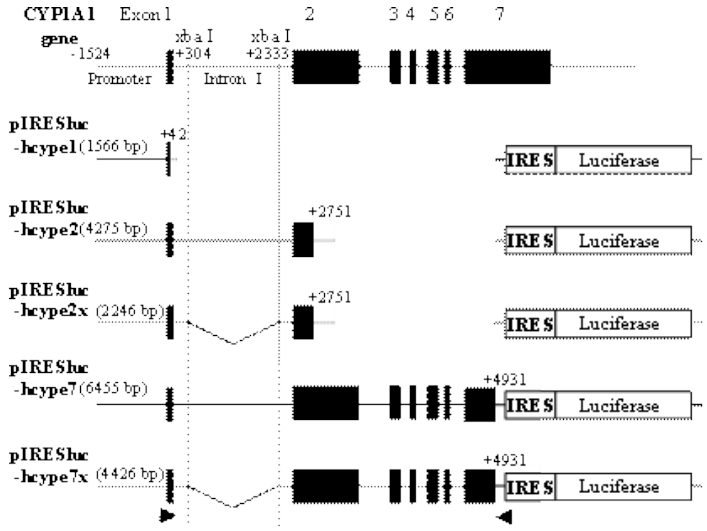

Figure 1. Human *CYP1A1* gene deletion constructs used for luciferase assay. DNA sequence were from GenBank™ accession number AF253322. ■ shows exons 1-7. Intron I encompassed the deletion sequence +305 ~ +2332. Filled arrowheads, the primers used to amplify the cDNA flagments.

Cell culture Chinese hamster ovarian cell lines (CHO-K1) were obtained from RIKEN Cell Bank (Tsukuba, Japan). The cells were cultured in F-12 Nutrient Mixture (Ham's F-12) medium with 10% fetal bovine serum at 37°C in a humidified air incubator supplemented with 5% CO_2.

Transfection and luciferase assay Transient transfection experiments were performed using Superfect® Transfection Reagent (QIAGEN, Hilden, Germany) on cells grown to 50% confluence in 48-well plates. CHO cells were co-transfected with 100 ng of each reporter construct, 250 ng of C57BL/6 mouse AhR expression vector (pCI-mAhR(B6)) and 250 ng of rat ARNT expression vector (pCI-rARNT). Three hours later, the transfection reagent was removed and cells were cultured with their normal growth medium. 24 hrs after transfection, cells were treated with 0 to 1 μM of 3-MC. For solvent contrast, cells were treated with DMSO (0.1% v/v). Following 24 hrs of incubation, luciferase activities were measured using the Luciferase Assay System (Promega) according to the manufacturer's instructions.

RT-PCR and Direct Sequencing CHO cells transfected with reporter and expression vectors as described above and also treated with 1 μM of 3-MC were used to isolate mRNA transcribed from reporter vectors. Total RNA was isolated from the cells by using the RNeasy Mini Kit (QIAGEN) and then treated with DNase I. Immediately after heating at 70°C, cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen, CA) with oligo dT primers. Diluted cDNAs were subjected to PCR amplification with a forward primer located on *CYP1A1* exon 1 (5'-TTCCCAGCTC AGCTCAGTAC CTCA-3') and a reverse primer located on 5'-end of IRES (5'-CCTTGTAGTC CTCGAGTTAC TCAC-3'). The PCR reaction was electrophoresed on a 1% agarose gel and the bands were excised to determine the sequences by using an Applied Biosystem 377-3100 Automated Sequencer and a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences Co., NJ).

Results and Discussion

All five constructs were connected to the IRES sequence via the internal *CYP1A1* exons. Three stop codons are presented just before of the IRES sequence, so as to translate a single luciferase protein. All constructs showed relatively lower basal levels of luciferase activity in the DMSO treated groups, but they were higher than background, indicating that transfection efficiency was even in all constructs (Fig 2). All five constructs represented induction of luciferase activities in a dose-dependent manner. pIRESluc-hcype1, which is most close to the conventional reporter vector showed the highest activity, and the lowest activity was shown by pIRESluc-hcype7. Compared with the complete constructs, the deletion of +305 ~ +2332 showed higher activity in both pIRESlu-hcype2x and pIRESluc-hcype7x. Certainly the distance between the *CYP1A1* promoter and luciferase gene affects the activity. Luciferase activity declined as the distance increased, but pIRESluc-hcype7x displayed more intensive luciferase activity than pIRESluc-hcype2 ($p<0.05$), even though the distance was longer. Furthermore, repeated experiments indicated the same tendency that pIRESluc-hcyp7x displayed a higher fold induction than pIRESluc-hcyp7 (data not shown). These results suggested that the deletion sequence had a slight suppression status for *CYP1A1* induction.

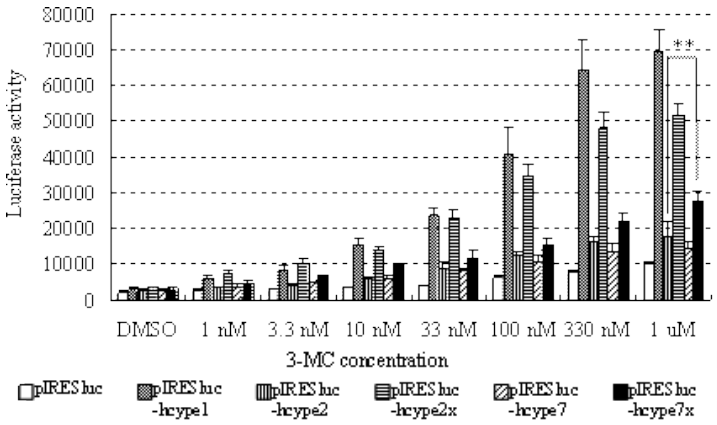

Figure 2. Dose-response curves of luciferase activities from the generated five constructs. CHO cells were cultured to 50% confluence in 48-well plates. Cells were co-transfected with each constructs, C57BL/6 mouse AhR expression vector (pCI-mAhR(B6)) and rat ARNT expression vector (pCI-rARNT), and then treated 3-MC or DMSO for 24 hrs. ** Significant difference ($p<0.01$)

To confirm whether introns were uniformly spliced or not, we isolated the total RNA of CHO cells transfected with each construct and then subjected to RT-PCR (Fig. 3). If the mRNA was spliced correctly, pIRESluc-hcype1 displays 68 bp, e2/e2x 430 bp, e7/e7x 1545 bp DNA fragments. Although pIRESluc-hcype1 showed no specific bands, maybe due to the fact that the fragment was too short to detect, pIRESluc-hcype2 and e2x displayed the same banding pattern, as well as e7 and e7x. Direct sequencings of the 430-bp bands revealed that both pIRESluc-hcype2 and e2x expressed the expected mRNAs spliced at normal splice sites. Two bands were detected in both pIRESluc-hcype7 and e7x. The upper 1545-bp band showed an expected sequence. The lower 1352-bp band was from an unexpected splicing that occurred within exon 2. Overall, these results suggested that the deletion of the internal sequence (approximately 87%) did not disturb the splicing of the first intron and also that similar CYP1A1 mRNAs were generated from the constructs.

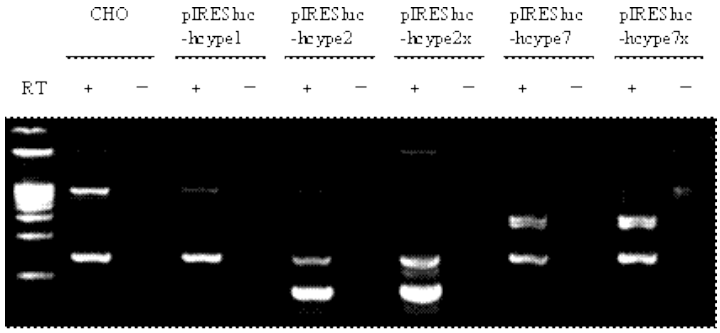

Figure 3. cDNA generated from total RNA of CHO cells which were transfected with five constructs were amplified by PCR. Marker (first lane) was 500bp DNA Ladder (TaKaRa Bio, Shiga, Japan). CHO cells were treated with 1μM 3-MC for 6 hours. Non-specific bands (about 700-bp, 4000-bp) were exist.

Most of cell lines showed super-induction of CYP1A1 transcription and mRNA by protein synthesis inhibitors.[3] Recently this phenomena has been hypothesized to be due to a putative labile repressor which might associate with DNA sequences that could be located further upstream, downstream, or within the *CYP1A1* itself.[2] Moreover, using mapping studies for DNase I hypersensitive sites in HepG2 cells, TCDF treatment detaches the binding protein of the first intron of the *CYP1A1* gene.[4] The results presented here might explain how these putative repressing factors interact with the first intron of *CYP1A1* gene. Further studies like a deletion analysis or a gel shift assay are needed.

In summary, luciferase activities of pIRESluc constructs suggest that the first intron of human *CYP1A1* gene may suppress the induction. The generated pIRESluc vector system might be useful to investigate the function of internal sequences of genes or the stability of primary transcript.

Acknowledgements

This work was supported in part by the Environmental Technology Development Fund (to S. O) from the Ministry of the Environment, Japan.

References

1. Uno, S., Dalton, T. P., Sinclair, P. R., Gorman, N., Wang, B., Smith, A. G., Miller, M. L., Shertzer, H. G., Nebert, D. W. (2004) *ToxicolApplPharmacol* 196, 410-421.

2. Monk, S. A., Denison, M. S., Rice, R. H. (2003) *Arch BiochemBiophys* 419, 158-169.

3. Lusska, A., Wu, L., Whitlock, J. P., Jr. (1992) *J BiolChem* 267, 15146-15151.

4. Gradin, K., Wilhelmsson, A., Poellinger, L., Berghard, A. (1993) *J BiolChem* 268, 4061-4068.